CD4+CD25+ Regulatory T Cell Isolation Kit
mouse

Order no. 130-091-041

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1. Description

Components

1 mL CD4+CD25+ Regulatory T Cell Biotin-Antibody Cocktail, mouse:
Cocktail of biotin-conjugated monoclonal anti-mouse antibodies against: CD8a (Ly-2; isotype: rat IgG2a), CD11b (Mac-1; isotype: rat IgG2b), CD45R (B220; isotype: rat IgG2a), CD49b (DX5; isotype: rat IgM) and Ter-119 (isotype: rat IgG2b).

2 mL Anti-Biotin MicroBeads:
MicroBeads conjugated to monoclonal anti-biotin antibody (isotype: mouse IgG1)

1 mL CD25-PE, mouse:
Monoclonal anti-mouse CD25 antibody conjugated to R-Phycoerythrin (PE) (clone: 7D4; isotype: rat IgM)

1 mL Anti-PE MicroBeads:
MicroBeads conjugated to monoclonal anti-PE antibodies (isotype: mouse IgG1)

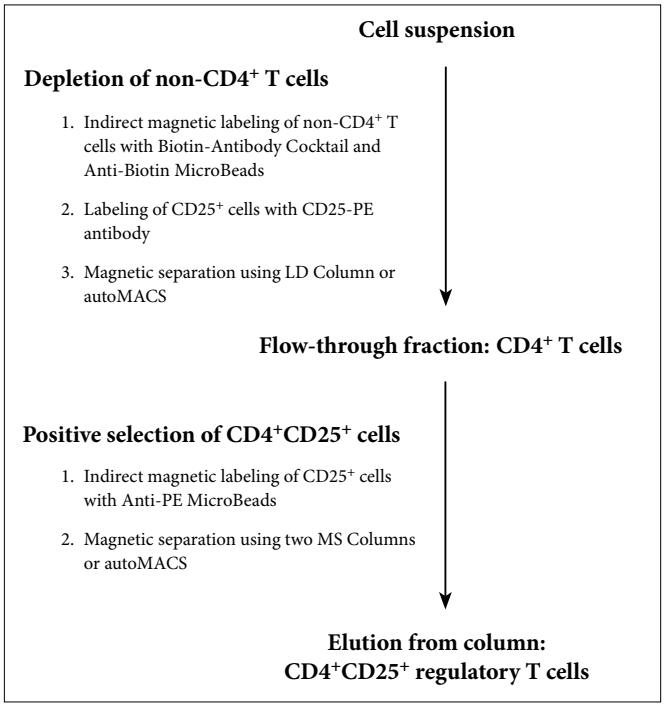
Size
For 10^9 leukocytes, up to 100 separations.

Product format
All components are supplied in buffer containing stabilizer and 0.05% sodium azide.

Storage
Store protected from light at 2–8 °C. Do not freeze. The expiration date is indicated on the vial label.

1.1 Principle of MACS® Separation

Mouse CD4+CD25+ regulatory T cells are isolated in a two-step procedure. First, CD4+ T cells are pre-enriched by depletion of unwanted cells. Then, CD25+ cells are positively selected from the enriched CD4+ T cell fraction.
For the isolation of CD4+ T cells, non-CD4+ T cells are indirectly magnetically labeled with a cocktail of biotin-conjugated antibodies and Anti-Biotin MicroBeads. In parallel, the cells are labeled with CD25-PE. The cell suspension is loaded onto a MACS® Column, which is placed in the magnetic field of a MACS Separator. The magnetically labeled non-CD4+ T cells are retained in the column, while the unlabeled, CD4+ T cells run through.
For the isolation of CD4+CD25+ cells, the CD25+ PE-labeled cells in the enriched CD4+ T cell fraction are magnetically labeled with Anti-PE MicroBeads. The cell suspension is again loaded onto a column which is placed in the magnetic field of a MACS Separator. The magnetically labeled CD4+CD25+ cells are retained in the column, while the unlabeled cells run through. After removal of the column from the magnetic field, the retained CD4+CD25+ cells are eluted as the positively selected cell fraction and once again separated over a new column, to achieve highest purities.


Cell suspension
Depletion of non-CD4+ T cells
1. Indirect magnetic labeling of non-CD4+ T cells with Biotin-Antibody Cocktail and Anti-Biotin MicroBeads
2. Labeling of CD25+ cells with CD25-PE antibody
3. Magnetic separation using LD Column or autoMACS
Flow-through fraction: CD4+ T cells
Positive selection of CD4+CD25+ cells
1. Indirect magnetic labeling of CD25+ cells with Anti-PE MicroBeads
2. Magnetic separation using two MS Columns or autoMACS
Elution from column:
CD4+CD25+ regulatory T cells

1.2 Background and product applications

The CD4+CD25+ Regulatory T Cell Isolation Kit was developed for the isolation of mouse CD4+CD25+ regulatory T cells from single-cell suspensions of spleen or lymph nodes.
CD4+CD25+ immunoregulatory T cells have been shown to actively suppress immune responses against autologous and foreign antigens

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in vivo and *in vitro*. CD25, the IL-2Rα chain, is also expressed on activated CD8$^+$ T cells, dendritic cells (DCs), and B cells.
The kit contains a cocktail of lineage specific biotin-conjugated antibodies against CD8 (Ly-2), CD11b (Mac-1), CD45R (B220), CD49b (DX5), Ter-119, and Anti-Biotin MicroBeads for depletion of non-CD4$^+$ T cells, as well as CD25-PE and Anti-PE MicroBeads for subsequent positive selection of CD4$^+$CD25$^+$ regulatory T cells.

Example of applications

- Isolation of CD4$^+$CD25$^+$ regulatory T cells from single-cell suspensions of spleen or lymph nodes for:
  - co-culture experiments with DCs to study priming of DCs for tolerance induction *in vitro* and after adoptive transfer of primed DCs *in vivo*.[1]
  - adoptive transfer experiments (e.g. from UV-exposed mice) to analyze the role of regulatory T cells during induction and elicitation of hapten-specific tolerance.[2]

1.3 Reagent and instrument requirements

- Buffer: Phosphate-buffered saline (PBS), pH 7.2, supplemented with 0.5% bovine serum albumin (BSA) and 2 mM EDTA. Keep buffer cold (4–8 °C). Degas buffer before use, as air bubbles could block the column.

  ▲ **Note:** EDTA can be replaced by other supplements such as anticoagulant citrate dextrose formula-A (ACD-A) or citrate phosphate dextrose (CPD). BSA can be replaced by other proteins such as gelatine, murine serum, or fetal calf serum. Buffers or media containing Ca^{2+} or Mg^{2+} are not recommended for use.

- (Optional) Pre-Separation Filters (# 130-041-407) to remove cell clumps.
- (Optional) Propidium iodide (PI) or 7-AAD for the exclusion of dead cells.
- (Optional) Fluorochrome-conjugated CD4 antibody, e.g., CD4-FITC (# 130-091-608), CD4-PE (# 130-091-607), or CD4-APC (# 130-091-611).
- MACS Columns and MACS Separators: Depletion of non-CD4 T cells is performed on an LD Column. The subsequent positive selection of CD4$^+$CD25$^+$ T cells is performed on two MS Columns. Depletion and positive selection can also be performed by using the autoMACS™ Separator.

Column	Max. number of labeled leukocytes	Max. number of total leukocytes	Separator
Depletion			
LD	10^8	5×10^8	MidiMACS, QuadroMACS, VarioMACS, SuperMACS
Positive selection			
MS	10^7	2×10^8	MiniMACS, OctoMACS, VarioMACS, SuperMACS
Depletion and positive selection			
autoMACS	2×10^8	4×10^9	autoMACS

▲ **Note:** Column adapters are required to insert certain columns into the VarioMACS™ or SuperMACS™ Separators. For details see the respective MACS Separator data sheet.

2. Protocol

2.1 Sample preparation

Prepare a single-cell suspension from spleen or lymph nodes using standard methods.

▲ **Note:** The Kit is not optimized for the isolation of regulatory T cells from blood and thymus.

▲ Red blood cell lysis or density gradient centrifugation is not necessary, since the CD4$^+$CD25$^+$ T Cell Biotin-Antibody Cocktail contains Anti-Ter-119 antibody.

2.2 Magnetic labeling of non-CD4$^+$ cells and fluorescent labeling of CD25$^+$ cells

▲ Work fast, keep cells cold and use pre-cooled solutions. This will prevent capping of antibodies on the cell surface and non-specific cell labeling.

▲ Volumes for magnetic labeling given below are for a starting cell number of 10^7 leukocytes. When working with fewer than 10^7 cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly (e.g. for 2×10^7 leukocytes use twice the volume of all indicated reagent volumes and total volumes).

▲ For an optimal performance it is important to obtain a single-cell suspension before magnetic separation. Pass cells through 30 µm nylon mesh (Pre-Separation Filters, # 130-041-407) to remove cell clumps which may clog the column.

1. Determine number of leukocytes.
2. Centrifuge cells at 300×g for 10 minutes. Aspirate supernatant completely.
3. Resuspend cell pellet in 40 µL of buffer per 10^7 total cells.
4. Add 10 µL of Biotin-Antibody Cocktail per 10^7 total cells.
5. Mix well and refrigerate for 10 minutes (4–8 °C).

   ▲ **Note:** Working on ice may require increased incubation times. Higher temperatures and/or longer incubation times may lead to non-specific cell labeling.
6. Add 30 µL of buffer, 20 µL of Anti-Biotin MicroBeads and 10 µL of CD25-PE antibody per 10^7 total cells.
7. Mix well and refrigerate for an additional 15 minutes in the dark (4–8 °C).
8. Wash cells by adding 1–2 mL of buffer per 10^7 total cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
9. Resuspend cell pellet in buffer:
   Depletion with LD Column: 500 µL for up to 1.25×10^8 cells.
   Depletion with autoMACS: 500 µL for up to 10^8 cells.

   ▲ **Note:** For larger cell numbers, scale up buffer volume accordingly.
10. Proceed to magnetic separation (2.3).

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2.3 Magnetic separation: Depletion of non-CD4$^+$ T cells

Depletion with LD Column

1. Place LD Column in the magnetic field of a suitable MACS Separator. For details see LD Column data sheet.
2. Prepare column by rinsing with 2 mL of buffer.
3. Apply cell suspension onto the column.
4. Collect unlabeled cells which pass through and wash column with 2×1 mL of buffer. Perform washing steps by adding buffer successively once the column reservoir is empty.
   Collect total effluent. This is the unlabeled CD4$^+$ T cell fraction.
5. Proceed to 2.4 for the enrichment of CD4$^+$CD25$^+$ T cells.

Depletion with the autoMACS™ Separator

▲ Refer to the autoMACS™ User Manual for instructions on how to use the autoMACS Separator.

1. Prepare and prime the autoMACS Separator.
2. Place the tube containing the magnetically labeled cells in the autoMACS Separator. Choose separation program "Depl025".
3. Collect the unlabeled fraction from outlet port neg1. This is the enriched CD4$^+$ T cell fraction.
4. Proceed to 2.4 for the enrichment of CD4$^+$CD25$^+$ T cells.

2.4 Magnetic labeling of CD25$^+$ cells

▲ Volumes for magnetic labeling given below are for an initial starting cell number of up to 10^7 leukocytes. For larger initial cell numbers, scale up volumes accordingly.

1. Centrifuge isolated CD4$^+$ T cells at 300×g for 10 minutes. Aspirate supernatant completely.
2. Resuspend cell pellet in 90 µL of buffer.
3. Add 10 µL of Anti-PE MicroBeads.
4. Mix well and refrigerate for 15 minutes in the dark (4–8 °C).
   ▲ **Note:** Working on ice may require increased incubation times. Higher temperatures and/or longer incubation times may lead to non-specific cell labeling.
5. Wash cells by adding 1–2 mL of buffer and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
6. Resuspend up to 10^8 cells in 500 µL of buffer.
   ▲ **Note:** For larger cell numbers, scale up buffer volume accordingly.
7. Proceed to magnetic separation (2.5).

2.5 Magnetic separation: Positive selection of CD4$^+$CD25$^+$ regulatory T cells

Positive selection with MS Columns

▲ To achieve highest purities, always perform two consecutive column runs.

1. Place MS Column in the magnetic field of a suitable MACS Separator. For details see MS Column data sheet.
2. Prepare column by rinsing with 500 µL of buffer.
3. Apply cell suspension onto the column.
4. Let cells pass through and wash column with 3×500 µL of buffer. Perform washing steps by adding buffer three times, once the column reservoir is empty.
5. Remove column from the separator and place it on a suitable collection tube.
6. Pipette 1 mL of buffer onto the column. Immediately flush out the magnetically labeled cells (CD4$^+$CD25$^+$ cells) by firmly pushing the plunger into the column.
   ▲ **Note:** To perform a second column run, you may elute the cells directly from the first onto the second, equilibrated column instead of a collection tube.
7. Repeat the magnetic separation procedure as described in steps 1 – 6 by using a new MS Column.

Positive selection with the autoMACS Separator

▲ Refer to the autoMACS User Manual for instructions on how to use the autoMACS Separator.

1. Prepare and prime the autoMACS Separator.
2. Place the tube containing the magnetically labeled cells in the autoMACS Separator. Choose separation program “Posseld2“.
3. Collect the positive fraction from outlet port pos2. This is the enriched CD4$^+$CD25$^+$ T cell fraction.

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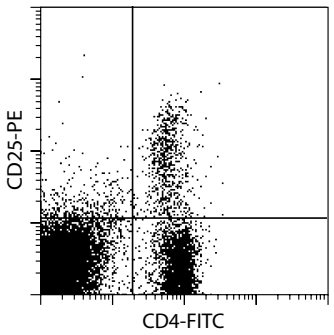
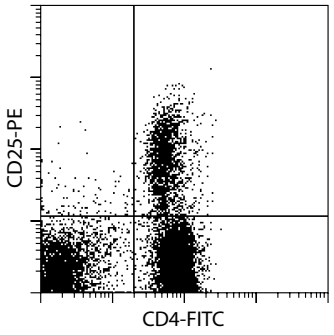
3. Example of a separation using the CD4$^+$CD25$^+$ Regulatory T Cell Isolation Kit

CD4$^+$CD25$^+$ regulatory T cells were isolated from mouse spleen cell suspension using the CD4$^+$CD25$^+$ Regulatory T Cell Isolation Kit, an LD and two MS Columns, a MidiMACS™ Separator and a MiniMACS™ Separator. Cells were additionally stained with CD4-FITC. Cell debris and dead cells were excluded from analysis based on scatter signals and PI fluorescence.

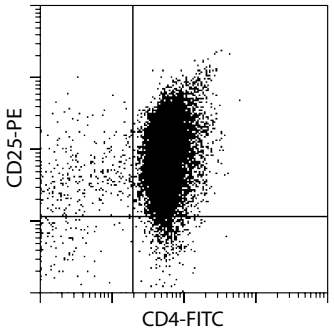
Spleen cells before separation



CD4$^+$ T cells after depletion of non-CD4$^+$ T cells



Isolated CD4$^+$CD25$^+$ regulatory T cells



4. References

1. Fallarino, F. *et al.* (2003) Modulation of tryptophan catabolism by regulatory T cells. Nat. Immunol. 4(12): 1206–1212. [3982]

2. Schwarz, A. *et al.* (2004) Ultraviolet Radiation-Induced Regulatory T Cells Not Only Inhibit the Induction but Can Suppress the Effector Phase of Contact Hypersensitivity. J. Immunol. 172: 1036–1043. [3791]

Warnings

Reagents contain sodium azide. Under acidic conditions sodium azide yields hydrazoic acid, which is extremely toxic. Azide compounds should be diluted with running water before discarding. These precautions are recommended to avoid deposits in plumbing where explosive conditions may develop.

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